METHYLENE VIOLET AND METHYLENE AZURE.*

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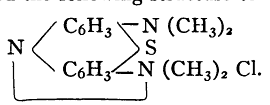
THE more recent advances in the knowledge of protozoal diseases have been associated with the development of methods of study, especially differential staining methods, and of these the Romanowsky stain deserves primary consideration. This stain, as is well known, depends upon the combination of eosin with altered methylene blue. There is, however, a lack of definite knowledge concerning it, as is at once apparent from the large number of empirical modifications offered in the literature. Certain of these modifications have attained a wide employment as general stains for histological examinations of blood. For these reasons it has seemed worth while to undertake a critical examination of our present knowledge of these stains and to advance by experimentation to more definite proof the various explanations which have been offered concerning the chemical composition of the ripened methylene blue solution and its mode of action.

Our more exact knowledge of methylene blue and its derivatives dates from the observations of Bernthsen,[1] who determined the composition of methylene blue chloride as $C_{16}H_{18}N_3SCl$, and assigned the following structure to it,



By treating methylene blue with dilute alkalis he obtained a mixture of bodies among which he recognized methylene violet, methylene azure, their leukobases and leuko-methylene blue. He was able to prepare pure methylene violet as follows. Twenty grams of recrystallized methylene blue iodide and four liters of water were placed in each of three flasks, warmed, filtered, and freshly precipitated silver oxide (made from 17 grams of silver nitrate) added to the warm filtrate from each flask, thoroughly shaken, and again filtered. The three solutions were then combined and diluted to 14 liters, and silver oxide, prepared from 25 grams silver nitrate, added, and again thoroughly shaken and filtered. The iodine-free filtrate was heated in several large flasks and kept boiling for one-half to one and a half days. The solution was very early filled with green shining crystals of mythylene violet while the vapor contained dimethylamine, which was collected and identified by its chemical reactions. The mother liquid at the end of the reaction contained only a little methylene violet, but considerable methylene azure, in solution. Leukobases were not recognized.

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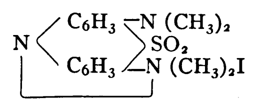
The precipitated methylene violet was filtered out and purified by repeated crystallization from alcohol, finally obtained as green, shining, needle-shaped crystals, almost insoluble in water, soluble in alcohol with a violet color and red fluorescence, in ether with a raspberry red color. It dissolved in warm dilute hydrochloric acid and reprecipitated on cooling as long dark needles. In concentrated sulphuric acid it dissolved with a violet blue color. These last two reactions are useful tests, the crystalline hydrochloride being obtained only with fairly pure methylene violet. Analysis indicated the formula $C_{14}H_{12}N_2SO$ which would be expected from its origin from methylene blue base by splitting off dimethylamine.

Methylene azure remained in the mother liquid after the precipitation of methylene violet by the prolonged boiling, together with a small amount of methylene violet. Bernthsen reduced the hot solution by means of stannous chloride and strong alkali. A half volume of alcohol was then added and the solution allowed to cool in a hydrogen atmosphere. The leuko-azure, crystallized out for the most part during the cooling, was filtered off in hydrogen atmosphere, washed in dilute alkaline stannous chloride solution and finally with pure water. It was then dissolved in dilute hydrochloride acid, oxidized by ferric chloride and salted out as a brown, amorphous precipitate containing some needle-shaped crystals. The chloride could not be purified, so potassium iodide was added to the solution and the iodide obtained in fine, green, shining needles and this repeatedly crystallized from hot water. The analysis indicated the formula $C_{16}H_{18}N_3SIO_2$, methylene blue plus two atoms of oxygen, with the following structure:



He was able to isolate methylene azure also by reduction of the solution as above and extraction with ether, carefully avoiding oxidation. The substance showed the following characteristics: its iodide was more soluble in water than methylene blue iodide, and fixed alkalis at once precipitated the free base and completely changed the color from blue to red, distinction from methylene blue; the chloride dissolved in concentrated sulphuric acid with green color, distinction from methylene violet.

Simon,[2] a student of Bernthsen who was engaged in this work, obtained results in the analysis of methylene azure indicating a distinctly different formula from the one finally selected by his teacher. He expressed himself as doubtful as to the purity of their methylene azure believing that it might still contain methylene violet. Some of the analyses upon which Bernthsen bases his formula were made by Simon.

Kehrmann and Schaposchnikoff[3] have shown that in methylene-blue salts the sulphur atom may be tetravalent and the linking of the orthochinon type instead of the parachinon of Bernthsen. Unfortunately these workers do not appear to have investigated methylene violet and methylene azure.

After this brief review of the purely chemical investigation of the action of alkalis on methylene blue, it is necessary to consider the researches of those more particularly concerned with the histological application of these dyes. The pioneers in this work were Romanowsky and Unna.

Romanowsky[4] in 1891 described a method of staining the hitherto unstained portion of malarial parasites by means of a mixture of aqueous solutions of methylene blue and eosin. The part of the parasite in question was stained in a carmin violet

color and recognized as the nucleus by Romanowsky. The staining was produced by adding an aqueous solution of eosin to an aqueous solution of methylene blue until a precipitate began to form. Simultaneously with the formation of this precipitate a new dye had been produced which had the elective affinity for the chromatin of the plasmodium. A slight excess of eosin or of methylene blue in the mixture was fatal to the nuclear stain. The exact ratio proved different with different brands of methylene blue, and even in the same solution it changed with age. Old methylene blue solutions covered with scum seemed to work best.

In the same year Unna[5] obtained his first red-stained mastzellen by accident when he was using an old solution of the composition,

Methylene blue	1.00 g.
Potassium hydroxide	0.05
Distilled water	100.00 c.c.

With a fresh solution he could not duplicate the result. There had been here a ripening. This ripening was not due to bacteria; hydrogen peroxide and carbonic acid failed to alter the fresh solution so that it would give such a stain. Therefore he concluded that the change must be due to the alkali. An old alkaline solution shaken with ether was found to color the latter an intense red (methylene violet). A solution rich in methylene violet was then made as follows:

Methylene blue	1 g.
Potassium carbonate	1
Alcohol	20 c.c.
Distilled water	100

Boil slowly on the water-bath until 100 c.c. remain. A dark violet solution resulted which has become widely known as Unna's polychrome methylene blue.

Ziemann[6] showed that certain brands of methylene blue could be used for Romanowsky staining when freshly made up, while others were useless for this purpose.

Nocht[7] conceived the idea that the nuclear stain might be due to some other dye present as an impurity in methylene blue, becoming active only when the eosin and methylene blue had precipitated each other. So he added some of Unna's polychrome methylene blue to the Romanowsky staining mixture and found that the red nuclear stain was improved. With the polychrome methylene blue and eosin alone he was unable to get any result until the alkalinity of the former had been considerably reduced by the addition of dilute acetic acid. Then the chromatin red was produced, but cytoplasm was not well stained until methylene blue was again added to the mixture. A mixture of neutralized polychrome blue, methylene blue, and eosin in water stained in a few hours, but did not overstain in 24 hours. Nuclei of the older forms of malarial parasites were not stained by this procedure. In a later note[8] he reported that if methylene blue solution be made weakly alkaline, heated a few hours in a steamer, filtered, neutralized, and added to unaltered methylene blue, the final mixture, having a clear blue color, would contain the essential nuclear stain; thus the use of Unna's polychrome methylene blue was dispensed with.

Nocht[9] subsequently showed that a new dye was developed in the ripened methylene blue, by extracting the solution with chloroform, which took on a red color. Evaporation of the chloroform at room temperature left a residue, "roth aus methylene blau," soluble in cold water with red violet color and neutral reaction. This *roth aus methylene blau* alone, in some experiments, stained the nuclei of young forms of malarial parasites, but not of the older forms. The same proved true of a solution

of *roth aus methylene blau* in methylene blue and of a mixture of *roth aus methylene blau* with eosin. When, however, all three dyes, methylene blue, *roth aus methylene blau*, and eosin were brought together, the nuclei of the parasites stained without exception a brilliant red, even in the crescent forms.

Alkaline solutions were found to stain more readily than neutral ones. He considered Unna's polychrome blue essentially a solution of *roth aus methylene blau* in methylene blue. Its alkalinity was found to be variable and Nocht emphasized, therefore, that it should be neutralized, always, in using it for Romanowsky staining. However, he no longer recommended this solution as he has prepared a better one by heating a solution containing 1 per cent methylene blue and ½ per cent sodium carbonate at 50° to 60° C. for two days. At the end of this time the solution is still pure dark blue in color, but contains sufficient *roth aus methylene blau* to give excellent chromatin staining. It is used by adding two or three drops of 1 per cent aqueous eosin to 2 c.c. of water and then adding the ripened blue solution slowly until the mixture becomes so dark that the eosin color is hardly to be recognized. Float the preparation five to ten minutes. If at the end of this time a second preparation be put on the same mixture, the specific chromatin stain no longer appears, or only faintly after a long time. Therefore the mixture should be used but once.

Concerning the chemical identity of *roth aus methylene blau*, Nocht could only say that it was not identical with either methylene red or methylene violet.

Other methods of developing the red nuclear stain have been devised, such as the adding of silver oxide or borax to methylene blue, but they all depend upon the principle of Nocht, that is, to set free the methylene-blue base and allow it to decompose, the process being hastened by heat in most instances. Slight modifications of Nocht's method have been widely used with good success.

Louis Jenner[10] made a compound of methylene blue and eosin by mixing alcoholic or aqueous solutions of these dyes. This substance he was able to prepare quite pure with melting-point of 227°, and he regards it as a linking of methylene blue (base) and eosin (acid). By dissolving it in methyl alcohol he made an excellent fixing and staining reagent for blood smears. His stain does not, however, contain the nuclear staining principle of Romanowsky, but his work bears an important relation to this subject, chiefly because he introduced this valuable way of using methyl alcohol, which was later adopted by Leishman.

Michaelis[11] first undertook to explain the chemical nature of the Romanowsky stain and gives a quite complete summary of Bernthsen's work bearing upon it. His own experiments are not detailed, but he has concluded that methylene azure and methylene violet are produced by the decomposition of methylene-blue base, in different relative quantities according to the conditions of the experiment. By the rapid oxidation of methylene-blue base (silver oxide method) methylene violet is formed in excess; but in the gradual decomposition of methylene-blue solution treated with alkalis, methylene azure is the chief product.

As a distinguishing test he gives the reaction with concentrated sulphuric acid. Methylene blue dissolves in this acid with a green color, in dilute sulphuric or hydrochloric acid with a blue color. Methylene azure also dissolves in concentrated sulphuric acid with green color. Methylene violet, on the other hand, never shows the green color but gives a blue color with the acid. The test is made by bringing a dilute solution of the dye into a test tube containing the acid (ring test). Through Ehrlich he obtained samples of methylene violet and methylene azure. With the former he

was unable to make stained preparations for the free base was so insoluble that, by a saturated aqueous solution, nuclei were only slightly tinted blue. The hydrochloride likewise proved useless. Methylene azure (hydriodide), on the other hand, stained nuclei in tissue sections an intense blue, mucous and mastzellen granules red. Upon testing Unna's polychrome methylene blue with concentrated sulphuric acid he obtained a green ring, which indicated that methylene violet was not present in great amount as he had expected on account of its almost absolute insolubility in water. Methylene *roth* is absent, as it is destroyed by alkalis, by the action of which Unna's polychrome methylene blue is produced. The green ring with sulphuric acid indicates that the dye is chiefly methylene azure. One might suppose that this green ring is produced by undecomposed methylene blue, but he diluted the polychrome methylene blue with water, added sodium hydroxide, extracted with ether, and found that the water became colorless below the ether, from which he concluded that no trace of methylene blue was present. Michaelis regards azure as the essential nuclear dye and was able to stain nuclei red with it alone, that is, without the presence of eosin. For staining he recommended the following (slightly modified Nocht's) solution which is for sale by Grübler under the name of *azur-blau.* Dissolve 2 grams of methylene blue in 200 c.c. of distilled water, add 10 c.c. of tenth normal sodium hydroxide and boil 15 minutes, cool, and add exactly 10 c.c. tenth normal sulphuric acid, filter. To use add one part of this solution to five parts of aqueous eosin solution, mix and stain the fixed preparation 15 minutes. It stains only where the smear is thin.

Reuter[12] and Leishman[14] independently conceived the idea of utilizing the precipitate formed by mixing Nocht's solution with eosin. Reuter[13] examined the ethereal extract of Nocht's solution (dry Nocht's *roth aus methylene blau*) and found that when dissolved in water and mixed with eosin it gave rise to no chromatin staining. When a few drops of acetic acid were added to the extract, it formed a precipitate with eosin which was filtered out and dissolved in alcohol. The resulting solution showed no chromatin staining properties upon dilution with water. Unna's polychrome methylene blue, which is rich in *roth aus methylene blau,* gave no precipitate with eosin until acetic acid was added and the precipitate thus obtained, dried and dissolved in alcohol, had no chromatin-staining properties. After repeated experimentation, Reuter concluded that Nocht's *roth aus methylene blau* cannot cause the chromatin stain in malarial parasites, that it is an indicator of the presence of the essential dye and should be regarded as an end product of the decomposition of methylene blue by alkali, whereas the nuclear stain is an intermediate product in this reaction. For the latter substance he suggests the name *A(lkali) methylene blau.* To make his staining solution Reuter precipitated Nocht's solution with eosin, washed the precipitate several times, and dried it in the dessicator. Two-tenths of a gram was dissolved in 100 c.c. ethyl alcohol and 2 c.c. anilin added. Thus prepared, the solution was permanent. To stain, he added 30 drops of this to 20 c.c. of water, immersed the preparation 20 to 30 minutes, sometimes three to four hours. Any precipitate on the preparation could be removed with absolute alcohol. Reuter regarded his dye as bearing no relation to Michaelis' methylene azure.

Leishman[14] prepared and used his staining solution as follows:

Solution A.—To 1 per cent solution of medicinally pure methylene blue in distilled water add 0.5 per cent sodium carbonate and heat at 65° for 12 hours, then allow to stand 10 days at room temperature.

Solution B.—Eosin extra B. A. Grübler one to one thousand aqueous solution.

Mix solutions A and B in equal amounts and allow to stand six to twelve hours stirring at intervals; filter and wash the precipitate thoroughly, collect, dry, and powder. Fifteen-hundredths gram is dissolved in 100 c.c. pure methyl alcohol to form the staining solution. It keeps perfectly (five months). The solution is a clear dark blue color and shows greenish iridescence by reflected light. To stain add three to four drops to an unfixed film, held in Cornet's forceps. After half a minute add six to eight drops of distilled water and mix by rotation. Allow to stain five minutes; then wash in distilled water and allow the water to remain on the specimen one minute. Examine in water, dry in air, and mount in balsam. The entire procedure requires seven to eight minutes. The after-treatment with water is an important part of the differentiation.

Wright[15] prepared the polychrome solution by adding 1 per cent of methylene blue to a ½ per cent sodium bicarbonate solution and steaming for one hour. When cold he precipitated this by the addition of a 1:1,000 eosin solution, collected the precipitate on a filter, and dried without washing. When thoroughly dry a saturated solution in pure methyl alochol was made, filtered and diluted with 25 per cent of methyl alcohol. The staining is carried out as in Leishman's method.

Giemsa[16] undertook the further investigation of the claim made by Michaelis that azure is the nuclear dye of the Romanowsky stain. Methylene violet (*Badische Anilin- und Soda-Fabrik*) alone showed no staining power in aqueous solution. He made a crystalline eosin compound with this methylene violet, dissolved it in alcohol, and attempted to stain preparations by diluting this solution with water. A rich precipitate resulted, but no staining. He next tested methylene azure. The crude dye (containing some methylene violet) gave no results worth mentioning, but with Höchst methylene azure pure, excellent stains were obtained. Yet this azure still contained traces of methylene violet. He therefore prepared chemically pure azure hydrochloride by a rather complicated process and obtained it as needle-shaped crystals from alcohol. A solution of this mixed with eosin gave most excellent Romanowsky stains, with the added advantage that the same mixture could be used repeatedly, provided only that an excess of azure was present. The preparations lacked only the blue color of the protoplasm, which was here more of a gray tone. Addition of an equal part of methylene blue to the azure did not detract from the chromatin staining, but produced the typical blue color in the protoplasmic portions of the preparation. Addition of methylene violet, however, prolonged the staining and caused a dirty precipitation on the film. Giemsa thus agrees with Michaelis that methylene azure is the essential and only red nuclear dye of the altered methylene blue, but he believes that the eosin also takes part in the chromatin staining.

The color reaction of dilute solutions with sulphuric acid, as given by Michaelis, Giemsa[17] found untrustworthy for the determination of the relative quantities of methylene azure and methylene violet in a mixture. A mixture might contain 40 per cent methylene violet and still not be distinguished from pure azure by this test. A better test, in his experience, has been to add a few drops of fixed alkali to a dilute solution of the substance in question and shake it thoroughly with ether. If azure alone was present the ether became bright scarlet red. If methylene violet was also present the ether became more or less violet; in the absence of azure a pure violet color. The color of the ether should be compared with that of ethereal solutions of known chemically pure azure and violet. If undecomposed methylene blue was also present the water below the ether remained blue, if absent this was colorless.

Giemsa does not describe his method of making methylene azure, but the substance may be purchased from Grübler as "Azur I (pur.)" for five marks a gram. The mixture of it with methylene blue, equal parts, can be had for one-half this price, as "Azur II." It is used as follows. An 0.8 per cent solution Azur II and a 0.05 per cent eosin (Höchst extra water soluble) are made up. Ten c.c. of the latter are mixed with 1 c.c. of the azure blue solution and the fixed blood preparation immersed for 15 to 30 minutes, washed in water, and examined, dried, and mounted in acid-free balsam.

After many attempts to make a good staining solution by treating methylene blue with alkalis, Giemsa has concluded that this is practically impossible, for, whatever the conditions, methylene violet was always produced and the amount of methylene azure was relatively small, especially when heat was employed. Nocht's *roth aus methylene blau* he found to be a mixture of methylene-violet and methylene-azure bases. No way has been found to avoid the production of methylene violet in these solutions or to get rid of it after its formation. He made a solution of the azure-eosin compound in pure methyl alochol, but only mediocre results were obtained with it while fresh, and upon standing it decomposed and failed to stain chromatin after a few weeks. He therefore condemned Reuter's stain which he considered to be a mixture of the eosin compounds of methylene blue, methylene violet, and methylene azure, the last alone being the active principle. In a later report Giemsa[18] has simplified his method somewhat and now recommends the following preparation:

Azur II-eosin	3.0 g.
Azur II	0.8

finely ground and sifted through a fine sieve into 250 grams glycerin at a temperature of 60° C., dissolve; then add 250 grams methyl alcohol at a temperature of 60° C. Shake and allow to stand 24 hours at room temperature, filter. To stain, one drop of this solution is mixed with 1 c.c. of water and the fixed preparation immersed in it. The preparations are best fixed by pure methyl alcohol, three to five minutes. The addition of a few drops of very dilute alkali carbonate to the staining mixture intensifies the chromatin stain.

Unna[21] undertook to ascertain the real constituents of his polychrome methylene blue, inasmuch as Michaelis had made the statement that it consisted essentially of methylene azure. He obtained samples of methylene azure and several of its salts from Giemsa and tested it upon tissue sections. He found that its staining power was not equal to that of his polychrome methylene blue. Only when methylene violet was added to the azure solution did he get good preparations, and he therefore still regards methylene violet as a most essential constituent of polychrome methylene blue He obtained the best results in staining tissue sections with the following mixture:

Azure carbonate (Giemsa)	0.25 g.
Potassium carbonate	0.25
Methylene violet (Bernthsen)	1.00
Glycerin and distilled water, equal parts, to	100.00 c.c.

Marino[22] has recently modified methylene azure so that it may be used in methyl alcohol solution. The formula follows:

Methylene azure	0.50 g.
Methylene blue	0.50
Water	100.00 c.c.

Add 0.5 per cent sodium carbonate and place for 24 to 48 hours at a temperature of 37° C. or better at a higher temperature. Then precipitate with eosin, determining by titration the exact amount of this to be added. Filter and obtain a powder; 0.04 gram of this is dissolved in 20 c.c. methyl alcohol. This is used the same as Leishman's solution except that 0.005 per cent eosin solution is used in place of the distilled water to dilute the alcoholic solution on the cover-glass.

In a recent paper Giemsa[20] opposes Marino's modification for the same reason that he condemned Reuter's stain. In this paper he announces that he will soon publish in *Les folia hematologica* a complete compendium of the literature bearing upon the Romanowsky stain.

May[23] has observed that blood preparations stained in ordinary methylene blue and eosin may be changed into particularly good Romanowsky preparations by after-staining with a solution of Giemsa's Azur I or with a ripened alkaline methylene-blue solution. For some details the latter solutions gave better results than the pure azure and he thought it possible that this might be due to some other decomposition product of methylene blue.

From the brief review of these papers it is apparent that the nuclear dye of Romanowsky's stain depends upon some of the products of the treatment of methylene blue with dilute alkali, the necessary change taking place more quickly at higher temperatures. These products are fairly well known, thanks to the pioneer work of Bernthsen and his pupils, but his method of preparing the two apparently most important ones is so difficult and expensive that few have had opportunity to test them. Although one worker has discovered a method of making methylene azure much better than that of Bernthsen, he has not chosen to share this important knowledge, so that investigators are still dependent upon the old difficult method. The other product, methylene violet, though easily made by Bernthsen's method, is difficult to purify, and those who have tested it are not agreed as to its properties.

The disputed question of the essential constituents and the action of the Romanowsky stain can be entered upon only after these constituents have been recognized and prepared in a pure condition. There is no agreement in the literature upon this question. Michaelis has identified the essential nuclear dye of the Romanowsky stain as methylene azure, while Reuter has convinced himself that another substance "A(lkali)methylene blau," different from azure, is the important substance. Unna appears to stand alone in claiming that methylene violet acts as a stain, and he has not shown any relation between it and the Romanowsky stain.

Pure methyl alcohol, as a solvent for the dyes, has proven very valuable in the hands of several observers, yet Giemsa, with chemically pure azure and its derivatives at his command, has failed to prepare a useful solution in pure methyl alcohol, and condemns all such solutions.

To attempt to reconcile, confirm, or refute the various conflicting experimental results reported, and to perfect the practical staining procedure has been the double purpose with which our experiments were undertaken. Although these ends have not been accomplished in the desired degree, yet some of the results obtained appear of such importance that their confirmation by others is desirable, and, if they stand this test, they should mark an advance in our knowledge of the Romanowsky stain.

EXPERIMENTAL.

The first experiment was an attempt to isolate and identify Nocht's *roth aus methylene blau*, as this certainly seemed to bear some relation to the Romanowsky stain. Extraction with ether by separating funnel proved unsatisfactory as the yield was too small, so a mechanical extractor, somewhat like the Soxlet apparatus, was constructed from glass (Fig. 1) for this purpose. To 300 c.c. of 1 per cent methylene blue (med. pur.), 1.5 grams of sodium carbonate crystals were added and the solution heated on a boiling-water bath one-half hour. It was then cooled and placed in the bulb of the extractor and extracted with ether. The ether, in passing up through the Nocht solution, took on a deep cherry-red color, and, as the extraction proceeded, a solid ring collected on the wall of the receiving flask at the surface of the boiling ether. The extraction was continued for 10 hours, at the end of which time the ether was coming away with a pale reddish-yellow tint and a considerable ring had already collected on the flask below.

The apparatus was disconnected, the ether poured off, and the extract dried in an air current and scraped out. It weighed 0.15 gram and was dark purple, almost black, in color. It was almost entirely insoluble in water; under the microscope the undissolved flakes appeared as dense black masses. Where the light penetrated they were seen to be red-purple in color. In warm, dilute hydrochloric acid the substance dissolved with a blue color to reprecipitate on cool-

ing as yellowish-black needles (hydrochloride). The substance dissolved in concentrated sulphuric acid with a violet-blue color. The last two reactions identified it as nearly pure methylene violet (Bernthsen). From this it would appear that the decomposition of methylene blue by sodium carbonate and heat produces considerable methylene violet and only small amounts of methylene azure and that Nocht's *roth aus methylene blau* is chiefly methylene violet base. A solution of this ethereal extract and eosin in methyl alcohol failed to stain nuclei appreciably, the red color being very faint, thus confirming Reuter, who failed to get chromatin to stain with the ethereal extract. But the addition of a small amount of methylene blue (med. pur.) to the solution made an excellent staining reagent, the nuclei taking the deep purple-red color characteristic of the Romanowsky stain.

The extraction by ether of a fresh, 1 per cent methylene-blue solution containing ½ per cent sodium carbonate removed only a slight amount of coloring substance, the ether showing only a faint pink tint. When instead of sodium carbonate, ½ per cent sodium hydroxide was added and the extraction performed in the absence of air (extractor), the methylene blue was almost entirely decomposed in 24 hours, retaining only a pale blue tint. A black precipitate was thrown down in it. In the receiving flask below a considerable amount of dark brown substance had been deposited, the ethereal extract. After drying in a current of air this was found to be very soluble in water; it failed to form crystals with hydrochloric acid. By the test given on p. 427, methylene violet, methylene azure, and methylene blue were easily recognized in it. The last was apparently

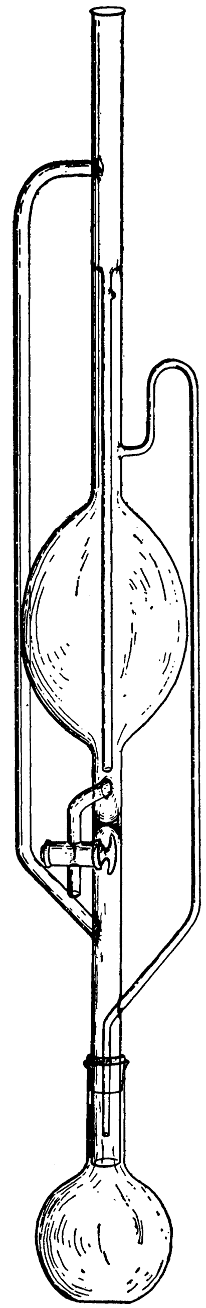


FIG. 1.

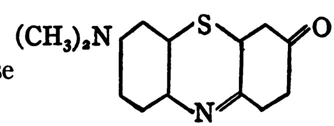
extracted as a leuko-base and oxidized during the subsequent drying.

Methylene violet was next prepared by Bernthsen's method (p. 412), purified by repeated crystallization from alcohol, and finally obtained as green, shining, needle crystals almost insoluble in water, soluble in concentrated sulphuric acid with a violet-blue color, and forming with dilute hydrochloric acid long needles soluble in warm, but insoluble in the cold dilute, acid.

A saturated alcoholic solution of this substance failed entirely to stain nuclei of blood cells when applied alone or diluted with water or watery eosin solution. In the presence of a little methylene blue it stained nuclei the characteristic purple-red color. After considerable experimentation this method of making methylene violet was simplified somewhat, and I now make it as follows:

Methylene blue (med. pur.)	4 g.
Sodium carbonate (crystals)	4
Distilled water	2,000 c.c.

Dissolve the sodium carbonate separately and add to the methylene-blue solution. Boil for five to ten hours, filter and wash the precipitate with water. It consists of long, needle crystals of methylene violet, often pure enough to form crystals with hydrochloric acid. This product I designate as crude methylene violet. It may be used for staining (see p. 427). There is considerable impurity, however, chiefly methylene blue and methylene azure, and to get pure violet it is necessary to recrystallize from alcohol repeatedly. In this way beautiful green, shining crystals are obtained. In making methylene violet from methylene blue, a dilute solution is essential, as otherwise the precipitate begins to form while there is still a large amount of undecomposed methylene-blue base present, and the product is correspondingly rich in this substance, from which it is quite difficult to separate the methylene violet.

Methylene violet base  , crystallizes from alcohol in opaque needles with a bright green luster, permanent in the air. It is insoluble in cold water, tints warm water a faint blue, dissolves slowly in ether with scarlet-red (cherry-red) color, not violet as

stated by Giemsa. It dissolves in methyl and ethyl alcohol with deep violet-blue color and rich red fluorescence. The formation of clean-cut needle crystals from alcohol is the best test for purity. Although insoluble in water, it is quite soluble in methylene-blue solution from which it may be extracted by ether even in the presence of an excess of carbonic acid. It is a weak base and appears to form no carbonate. It forms no compound with eosin in alkaline or neutral solutions, but in faintly acid solutions it forms a red-purple compound insoluble in water. This is at once decomposed by dilute sodium carbonate solution with formation of the free methylene-violet base. In concentrated sulphuric acid methylene violet dissolves with a violet-blue color, in strong hydrochloric acid likewise with a blue color. In fairly dilute (20 per cent) hydrochloric acid it dissolves when warmed and crystallizes out in long needles as the hydrochloride upon cooling. These crystals are soluble in water and in concentrated hydrochloric acid.

Methylene-violet hydrochloride was prepared by dissolving the pure base in warm, 1 per cent hydrochloric acid, filtering, and precipitating the dye by the addition of sodium chloride. The precipitate was washed on the filter with a saturated solution of sodium chloride several times and finally with pure water. Apparently the substance dissociated readily in the absence of free hydrochloric acid, for a strong aqueous solution gave a deep red color to ether without the addition of alkali. The crystalline form was also destroyed to a certain extent by washing with the salt solution. It was dried at a temperature of 55° C. *in vacuo* and finally obtained as a dark brown powder, soluble in water with a red-violet color, but immediately precipitated as amorphous red flakes by the addition of sodium carbonate.

An important property of methylene violet is that of combining with methylene blue. If 0.5 gram methylene violet and 0.5 gram methylene blue be dissolved in 100 c.c. of water by means of a few drops of hydrochloric acid and this solution then made slightly alkaline with sodium carbonate, it at once fills with crystals and the solution becomes quite pale violet in color. These crystals appear the same as those formed by boiling methylene blue with sodium carbonate in dilute solution, and consist of a combination or intimate mixture

of methylene violet and methylene blue. They are slightly soluble in water with a violet color. When a larger proportion of methylene blue is used as, for example,

Methylene-violet hydrochloride	0.2 g.
Methylene blue	0.8
Distilled water	100.0 c.c.

the addition of sodium carbonate no longer causes precipitation and the solution remains deep blue in color.

An attempt was also made to obtain methylene azure of Bernthsen. The filtrate from the preparation of methylene violet by Bernthsen's method was rendered slightly acid and then stannous chloride and alkali added until the color was completely reduced and the reaction strongly alkaline. It was then placed in the bulb of the mechanical extractor and extracted with ether. This fulfilled quite well the conditions given by Bernthsen, as the solution was completely protected from air. The ether came away colorless after the apparatus had been in operation a short time, but when this colorless ether was exposed to the air, it became red and a blue precipitate was deposited on the glass. Evidently the ether contained a substance which, when oxidized, became insoluble in ether. This led me to believe that the extract contained leukomethylene violet and leukomethylene blue, the latter precipitating as methylene blue upon exposure to the air while the violet remained in solution as the red color. The extraction was continued, and finally the ether poured out of the receiver and the deposited extract was dried by a current of air. During this process it probably took up oxygen and carbon dioxide. It was then found to be very easily soluble in water; dilute alkalis (fixed) caused immediate change from blue to purple color in the solution with precipitation of the color base; it failed to form crystals with hydrochloric acid and thus corresponded to some extent to the descriptions of methylene azure. Yet it was certainly mixed with methylene violet. Another lot of the solution was reduced and extracted in the same way, the receiving flask being changed after the first hour. It was found that the first portion was fairly rich in the red coloring substance, while that in the second flask was very poor in this color, its watery solution changing color somewhat upon the addition of alkali, but remaining much more blue than in the case of the first. This showed that the

ethereal extract varied in its qualitative composition, which would of course, be impossible if it contained a single substance. It evidently consisted of methylene azure, methylene violet, and methylene blue, extracted by ether as their leuko-bases. The separation of methylene azure from methylene violet gave Bernthsen and Simon considerable trouble, and Simon considered it quite possible that their purest azure still contained methylene violet. Giemsa also found that the yield of azure was very small by Bernthsen's methods of preparation. Pure methylene azure has been made by this investigator, but inasmuch as he has not published his method, it is impossible to follow his work. From his papers one would judge that his method of making it is a fairly complicated and expensive one.

Accepting the composition of methylene azure given by Bernthsen, I attempted to make it by treating methylene blue with hydrogen peroxide in acid and alkaline solutions, and with hydrogen peroxide and ferric chloride in acid solution, without result. By the use of chromic acid in acid solution, however, indications of azure formation were obtained at the first trial, as was also the case when potassium permanganate and acid were used. The following method was finally decided upon, and I shall endeavor to describe it so that anyone with a little chemical training can make pure methylene azure. Thirty-five c.c. of hydrochloric acid (Sp. G. 1. 20) is diluted to a liter with distilled water and 10 grams of Ehrlich's rectificiert methylene blue added and dissolved by heat. To the boiling solution 35 c.c. of a hot, 10 per cent aqueous solution of potassium bichromate is slowly added with constant stirring. A precipitate of methylene blue chromate forms in the solution, but as the boiling continues this redissolves with a deep blue color. The boiling is then continued until all the methylene blue has been converted into azure, as determined by the test here given. After ten minutes' boiling a few drops of the solution are removed to a test tube and diluted slightly with cold distilled water. A dilute solution of sodium hydroxide is then added drop by drop, shaking the test tube until the color has changed from blue to red. About 5 c.c. of ether is then added and the tube vigorously shaken a few moments and then allowed to settle, when the ether will have taken up the azure base and, if methylene blue is present, a blue tint will remain in the water below. The test should

be performed quickly, else a small amount of methylene blue may not be detected. This test is repeated at short intervals until methylene blue is no longer present. Then the hot solution is filtered and nearly saturated with sodium chloride and allowed to stand for about two hours. The azure hydrochloride precipitates as irregular bunches of small plate crystals, which are filtered out and recrystallized from alcohol to purify. To do this the precipitate is placed in a flask and a half liter of alcohol added, thoroughly mixed, heated to boiling on the water-bath, and filtered. The filtrate is then boiled, distilling off the alcohol until the dye begins to separate out of the boiling solution, whereupon it is poured out into a clean beaker and set aside to crystallize. These crystals are then filtered out, redissolved in fresh alcohol, and the operation repeated. It is finally obtained as long, hairlike crystals of a green color which become brown upon drying *in vacuo* at a temperature of 55° C. The yield is about three grams. When dissolved in water, treated with a drop of dilute alkali and extracted with ether, pure azure leaves no blue color in the water below. If the supernatant ether, containing the azure base is poured off into a tube containing sodium bicarbonate solution and shaken with this, the ether is decolorized and the water becomes blue. The base has been changed to the carbonate and as such is insoluble in ether.

I have not undertaken an ultimate analysis of this substance, but its origin from methylene blue by action of oxidizing agents, its correspondence in properties with the description given by Bernthsen, and its identity in chemical and staining reactions with "Azur I pur.," Giemsa, purchased from Grübler, led me to believe that it is methylene azure. The amount of bichromate used in making it is just slightly in excess of the theoretical amount required to furnish two atoms of oxygen to each molecule of methylene blue and this agrees with Bernthsen's analysis according to which the composition of the iodide is $C_{16}H_{18}N_3SIO_2$.

One of the most characteristic properties of methylene azure salts is the immediate complete change of color, upon the addition of free alkali, from blue to red, due to the setting free of methylene azure base, which is only very slightly soluble in water. This is a much stronger base than methylene violet, rapidly taking up carbonic acid

from the air to form a carbonate which is quite soluble in water with a blue color. Upon this property is based the method of detecting and separating methylene violet and methylene azure when present in the same solution (see below).

Methylene azure, like methylene blue, decomposes when heated with dilute alkali carbonates, with the formation of methylene violet and some other, undetermined substances. Methylene azure 0.5 gram, sodium carbonate 0.5 gram, distilled water 200 c.c., were heated over the direct flame and boiled 15 minutes. Even before the boiling point was reached a precipitate had begun to separate, and at the end of 15 minutes' boiling, the solution was quite pale. It was cooled somewhat and filtered, the filtrate giving a strong test for methylene violet. The precipitate consisted chiefly of thin crystals and dissolved partly in warm, dilute hydrochloric acid. This solution upon cooling formed long, needle crystals, methylene-violet hydrochloride.

Methylene azure-eosin may be formed by mixing strong alcoholic solutions of azure hydrochloride and sodium eosin. It crystallizes as fine purple needles, insoluble in water, slightly soluble in alcohol. It is also formed by mixing water solutions of these two dyes. Recrystallization from alcohol failed; apparently boiling alcohol decomposes the substance.

Methylene azure and methylene violet are both present in polychrome methylene blue, and as free bases both are soluble in chloroform with a deep red color. In ether, methylene-violet base has a deep pinkish-red color, raspberry red; azure base dissolves with a yellowish-red or brownish-red color. The latter is a strong base, combining readily with the carbon dioxide of the air to form a carbonate. Methylene violet-base, on the other hand, is set free from its salts by sodium bicarbonate or by calcium carbonate, and may then be extracted by ether. To test a given solution of ripened alkaline methylene blue for the presence of these substances, the method of Michaelis is insufficient, and the test given by Giemsa is inaccurate. The color of methylene violet in ether is not violet, but scarlet-red. The following test is an improvement. Dilute the unknown solution until it is nearly transparent in a test tube, add a few drops of 1 per cent hydrochloric acid and then an excess of solid sodium bicarbonate. Carbon dioxide should be evolved. If no bubbles appear, a few more

drops of the acid should be added. The addition of the acid serves to neutralize any previous free alkali in the solution, either free dye base, alkali hydroxide, or carbonate, and to saturate the solution with carbonic acid. The solution is now shaken with ether and a red color indicates methylene violet. By repeatedly extracting with fresh quantities of ether this is entirely removed. If now an alkali hydroxide is added to the solution and the ether extraction repeated, it now removes methylene azure, which has been held back in the water as a carbonate, and assumes an eosin-red color (yellowish in dilute solution). If methylene blue is absent the water is now completely decolorized, if present the water remains blue-tinted for a time. The test is not sharp here, for even dilute alkalis decompose the methylene blue so that a small quantity of this dye in the original solution may escape detection by this method. In doubtful cases it is well to use a fairly strong solution of the unknown and rapidly to extract both the red bases together (by adding alkali hydroxide and shaking with ether) and then note the color of the water. When properly performed one can easily detect all three dyes in Unna's polychrome methylene blue.

Methylene violet* may be used as a stain in either alcoholic or aqueous solutions. Alone, however, it does not stain as has been abundantly shown by previous workers. A saturated solution in methyl alcohol used as such, by diluting with distilled water, or with eosin solution shows no staining power. If, however, an equal amount of methylene blue be present the characteristic colors of the Romanowsky stain are produced. The following solution has proven quite satisfactory for general blood staining.

Methylene violet (pure crystals)	0.08 g
Methylene blue (med. pure)	0.16
Eosin (water soluble yellowish) Grübler	0.20

Powder and mix thoroughly and dissolve in 100 c.c. of warm pure methyl alcohol. Cool to room temperature, filter and dilute with 10 c.c. methyl alcohol. This solution is used as a fixing and staining

[1] The dye list of Schultz and Julius, Berlin, 1897, mentions methylenviolett, $C_{20}H_{19}N_4Cl$, dimethylsafranin chloride. Several brands of this substance are on the market under this trade name, methylenviolett, and they have been examined. It is a substance entirely different from Bernthsen's methylene violet and should not be confused with it. Recently two samples of the latter have been obtained. One bearing the label "Methylenviolett v. Bernthsen, Dr. G. Grübler & Co., Leipzig," is evidently an altered methylene blue, strongly alkaline, but contains only a trace of methylene violet. The other sample, labelled "Bernthsen's Insol Methylene Violet, E. Leitz, New York and Chicago," yields crystals with dilute hybrochloric acid and seems to be about as pure as thc crude methylene violet which I have prepared. In methyl alcohol solution made up according to the formula given, it is a satisacfory stain.

solution according to Leishman's method. I have experienced very little trouble from its running off the coverglass during the staining. For this purpose it has been found convenient to bend the blades on the Novy forceps so as to eliminate the tendency of the stain to run up between them. It can be done easily by anyone; the figure explains itself. The bend is so slight that the forceps may still be used

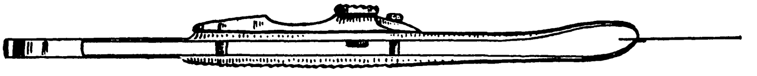


FIG. 2.

to pick up coverglasses from a flat surface. This staining solution seems to keep indefinitely in a well-stoppered bottle and, if by evaporation it becomes too concentrated, a little methyl alcohol may be added from time to time. I have used one solution at intervals for three months and it is still as good as when first prepared.

Pure methylene violet is not necessary to make a good methyl alcohol stain, for I have obtained good results by using the crude violet (see p. 427) which anyone can easily make. It should be quite dry before use. The following formula has given good results:

Methylene violet (crude)	0.08 g.
Methylene blue (med. pur.)	0.08
Eosin (water soluble yellowish)	0.20

These are dissolved in 100 c.c. methyl alcohol, filtered, and diluted as in the previous solution. The violet used here contains some methylene blue, traces of azure, and other undetermined substances.

These solutions, it seems to me, furnish the Romanowsky stain in the most convenient, surest, and best form for the use of physicians and clinical laboratories.

In staining films from syphilitic lesions, a very dilute solution of sodium carbonate (1:15,000) should be substituted for the distilled water ordinarily used to dilute the stain in Leishman's method.

For aqueous solution the methylene violet hydrochloride is employed, being mixed with an excess of undecomposed methylene blue and then rendered alkaline and allowed to stand a few days before using. For staining malarial parasites and trypanosomes the following solution has proven of value:

Methylene violet hydrochloride	0.1 g.
Methylene blue (med. pur.)	0.9
Glycerin	50.0 c.c.
Distilled water	40.0

Dissolve and add 10 c.c. of a 5 per cent sodium carbonate solution After standing a few days to allow the alkali to set free the dye bases the solution is used in the same way as that of Nocht, by adding a few drops to 2 to 10 c.c. of a very dilute (1:10,000) solution of eosin. The The exact proportions need to be determined by experiment, and therefore the method is not recommended for clinical work. For staining spirochetes a solution stronger in methylene violet has, so far, seemed better.

Methylene violet	0.2 g.
Methylene blue	0.8
Glycerin	50.0 c.c.
Distilled water	40.0

Dissolve and add 10 c.c. of 5 per cent sodium carbonate.

The use of these solutions presents the same difficulties met with in using Nocht's solution, but the staining is better. I hesitate to recommend them as I think the best combination has not yet been attained. Some surprisingly good preparations have been obtained with them, however. The preparations must be fixed before staining, either in absolute alcohol and ether, or in pure methyl alcohol (three to five minutes) as suggested by Giemsa. Preparations of spirochetes stain more intensely if placed for a few minutes in a 0.05 per cent solution of sodium carbonate and then rinsed just before staining. One may gain some idea of the intensity of the stain from the fact that often *Sp. Obermeieri* may be easily seen with the low power (Leitz Obj. No. 3, Oc. No. 1) after it is stained by this method. A tendency to formation of precipitate upon the preparation is a drawback, yet in some of the most intensely stained preparations this is absent.

The mode of action of methylene violet is somewhat problematical, but it seems most probable that the real dye penetrates and stains as a combination of methylene blue and methylene violet, "A(lkali) methylene blau" of Reuter, and that the methylene-blue constituent is then gradually removed by the action of eosin, leaving finally the red violet-base in the nucleus. In accord with this is the fact that methylene violet forms no combination with eosin in alkaline solution, and staining takes place only in alkaline solution.

These stains have been tested upon human malarial blood, blood of leukemia, pernicious anemia; blood from rats, mice, and guinea-pigs infected with *Trypanosoma Brucei;* from rats with *Tr. Lewisi*, from rats infected with *Sp. Obermeieri;* upon smears from primary and secondary luetic lesions.

The use of methylene azure in alcoholic solution has been found impracticable by Giemsa. I have tried the following solution:

Methylene azure hydrochloride	0.1 g.
Methylene blue (med. pur.)	0.1
Eosin, water soluble, yellowish	0.1
Methyl alcohol	100.0 c.c.

Much of the dye remained undissolved on the filter. The resulting solution gave fairly good stains of blood elements, malarial parasites and trypanosomes, not so good in the case of spirochetes. The solution gradually deteriorated, until at the end of two months no nuclear stain could be obtained with it in the ordinary way, only the red blood cells being stained with the eosin. But the substitution of very dilute sodium carbonate solution (1:10,000) for distilled water as the diluting fluid produced fairly good nuclear staining again. The gradual decomposition of the azure-eosin in methyl alcohol would seem, therefore, to give rise to acid, quite probably formic acid from oxidation of the solvent, and the deterioration of the solution is due to the fact that this acid prevents the dye from acting and not to the decomposition of all the azure-eosin, as has been supposed by Giemsa.

Marino's modification (p. 418) contains some alkali in the solution as made up, and so does not become useless so quickly. It will probably be found to deteriorate with age, however, as not all the azure has been decomposed by his preliminary heating.

With methylene azure in aqueous solution it is possible to obtain excellent stains of hematozoa. The various methods have been quite thoroughly tested by Giemsa, and I have gone into this part of the work only far enough to convince myself that his claims for azure are well founded. The preparations are remarkably free from precipitation. The color of the nuclear stain produced is, however, not exactly the same as that obtained by the method of Nocht or the numerous modifications of it. Certain details are brought out better by azure than by the older procedures or by methylene violet-blue solutions;

but certain other details are not, for example, the blood platelets and spirochetes. The following watery solution has proved very useful:

Methylene azure hydrochloride, pure	0.5 g.
Methylene blue (med. pur.)	0.5
Sodium carbonate	0.25
Glycerin	50.0 c.c.
Distilled water	50.0

The constituents dissolve readily. The solution is used by adding a few drops to 10 c.c. of very dilute (1:5,000) eosin solution and immersing or floating the fixed preparation on the mixture for 10 to 15 minutes. The resulting stain is excellent. Giemsa believes that the nuclear color is a combination of azure and eosin, while Michaelis regards it as due to the azure alone. I am inclined to regard Michaelis' view as the more correct, for a solution of methylene azure alone, rendered slightly alkaline with sodium carbonate, stains nuclei of leucocytes the red-purple color. Differentiation of detail is, however, not so good as when eosin is also used.

In general, methylene azure does not seem to stain chromatin so intensely nor to outline it so sharply as do the violet-blue solutions. Further experimentation is necessary to determine the best conditions for staining with the latter, especially in aqueous solutions. Mixtures of methylene violet with methylene blue and azure have been tested, but so far seem to offer no advantages.

The composition of ripened-alkaline methylene blue must be regarded as a variable one, both methylene violet and azure being among the constituents. If ripened at a high temperature very little of the latter is present, and methylene violet is the prominent staining factor. This is the case with most of the Romanowsky staining solutions recommended in the literature. By ripening at room temperature both the red dyes are produced.

A mere trace of methylene violet in a methylene blue solution is sufficient to cause the red chromatin stain when the solution is combined with eosin in the proper proportion. For instance a solution of

Methylene violet hydrochloride	0.005 g.
Methylene blue (med. pur.)	0.995
Sodium carbonate	0.25
Distilled water	100.00 c.c.

if combined with the proper amount of dilute eosin gives the red chromatin stain. The staining process requires about an hour.

In conclusion I take great pleasure in acknowledging my indebtedness to Professor G. Carl Huber, Director of the Histological Laboratory, for the incentive to undertake this .work and for constant counsel and encouragement during its progress. I am also under obligations to Professor F. G. Novy for important suggestions and the loan of valuable apparatus and materials, and for the blood parasites used in testing the stain, and to Dr. H. A. Freund for films of pathological human blood.

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